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**Sent:** Tuesday, June 03, 2003 7:52 AM
**To:** STIC-ILL
**Subject:** Papers for Examination of SN 09234290

Hi

I need the following papers to examine 09/234,290, this is a RUSH since this case is due this biweek.

1. Yoon et al, Annals of the NY Academy of Sciences, 2001, 928:200-211

2. Poulton et al, Diabetes/Metabolism Research and Reviews, 2001, 17(6)429-435 *****

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7. Seddon et al (Biochem Soc. Transactions,1997, 25(2)620-624)

8. Reddy et al, Histochemical Journal, 2000, 32(4)195-206

9. Ylinen et al, Pancrea, 2000, 20(2)197-205

10. Sainio et al, Pancrea, 1999, 18(3)282-293

11. Alamunits et al, Clinical and Experimental Immunology, 1999, 115(2)260-267.

I also need an entire volume, Cohen et al (Autoimmune Disease Models, A Guidebook, Academic Press, San Diego, 1994

Thanks
Susan Ungar
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703-305-2181
CM1-8B05

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# Role of $CD4^+CD8^-$ thymocytes in the prevention of autoimmune diabetes

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## Introduction

The immune system has evolved a number of potent mechanisms for the elimination of foreign pathogens. As these mechanisms are potentially damaging to the host, an essential feature of the adaptive immune response is that it should be able to distinguish self from non-self and respond, when appropriate, only to the latter. The state of non-reactivity of immune effector functions with host tissues is termed self tolerance and is maintained at all levels by specific mechanisms. Furthermore the failure of these mechanisms can result in serious immune-mediated tissue damage with the resultant pathology causing so-called autoimmune diseases. Diabetes, arthritis and multiple sclerosis are some examples of diseases in which an autoimmune mechanism has been implicated in their pathogenesis.

Abbreviations used: TxX rats, rat subjected to thymectomy and irradiation; $T_{reg}$, regulatory T-cells; $T_{diab}$, diabetogenic T-cells.

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## Passive and active mechanisms of self tolerance

With regard to T-cell tolerance, the immune system has evolved a number of strategies for preventing autoreactive T-cells from making potentially pathogenic responses against host tissues. The widely accepted mechanisms of self tolerance can be classed as passive mechanisms and function both intrathymically and extrathymically. During T-cell development in the thymus, T-cells bearing receptors that recognize complexes of major histocompatibility antigens and self-peptides with a high affinity are induced to die [1]. In the periphery, foreign antigen is presented to specific T-cells by antigen-presenting cells which can provide important co-stimulatory signals for T-cell activation, whereas it is considered that self-antigens are presented by cells lacking the ability to provide such signals [2,3] with the result that potentially autoreactive T-cells are either rendered anergic [2] or simply do not respond to antigen [4]. These mechanisms are termed passive because they rely upon a functional absence of autoreactive cells, either through their physical deletion intrathymically or

their functional inability peripherally. However, there are now data from many studies that strongly suggest that these mechanisms cannot explain tolerance to all self-antigens. These studies have demonstrated the existence of overtly autoreactive T-cells that have neither been deleted nor are anergic, but whose control requires the presence of a population of regulatory T-cells [5–10]. It is these so-called active mechanisms of self tolerance that are the subject of our studies.

## Induction of diabetes in a normal strain of laboratory rat

Previous studies in this laboratory showed that a normal strain of laboratory rat, PVG.RT1$^u$, with no predisposition to spontaneous autoimmune disease can be induced to develop autoimmune diabetes by a protocol of adult thymectomy and four doses of 250 rad $\gamma$ irradiation at 2-week intervals after thymectomy (TxX). Disease onset starts around 3 weeks after the last irradiation, with 70% of females and almost 100% of males developing diabetes during the ensuing 10-week period [9]. Extensive mononuclear infiltrates, including T-cell receptor expressing cells, were observed in the pancreas of TxX rats, and destruction of $\beta$-cells was shown to be dependent on CD8$^+$ T-cells since their depletion prevented disease onset. Reversal of the lymphopenia of TxX rats by their reconstitution with thoracic duct lymphocytes from normal syngeneic donors prevented diabetes development in 50% of recipients. Fractionation of thoracic duct lymphocytes into CD4$^+$ and CD8$^+$ T-cells revealed that protection from diabetes could be wholly attributed to the CD4$^+$ cells, also protecting 50% of recipients.

The use of the monoclonal antibody OX22, specific for the C exon of CD45, has proved useful in the fractionation of peripheral CD4$^+$ T-cells into functionally distinct subsets. CD4$^+$CD45RC$^+$ T-cells bear some of the characteristics of Th1-like cells since they secrete high levels of interferon $\gamma$ and interleukin 2 after activation *in vitro* [11,12], and there is evidence that they contain cells with an autoreactive potential, since their adoptive transfer into nude recipients results in a wasting disease characterized by mononuclear infiltrates in many organs [13]. In contrast, CD4$^+$CD45RC$^-$ cells are a potent source of interleukin 4 after activation *in vitro* [12], and their co-transfer with CD4$^+$CD45RC$^+$ cells into nude recipients prevented the induction of wasting disease by the latter population [13]. When assayed for their ability to prevent diabetes, it was found that a dose of $5 \times 10^6$ CD4$^+$CD45RC$^-$ cells was sufficient to prevent development of diabetes in all recipients, whereas no rats receiving $5 \times 10^6$ CD4$^+$CD45RC$^+$ cells were protected from diabetes development [9].

## Thymocytes are more potent than peripheral cells at preventing autoimmune diabetes

CD4$^+$CD45RC$^-$ cells are a heterogeneous population consisting of at least three different subsets. Further fractionation of this population revealed the phenotype of the protective cells to be CD4$^+$CD45RC$^-$ Thy-1$^-$RT6$^+$TCR$\alpha\beta^+$ [9], which is the phenotype of a resting primed cell. The implication of this result was that the regulatory T-cell responsible for the protection of TxX rats from development of diabetes was generated in the periphery by clonal expansion of naive cells after encounter with an antigen of unknown specificity. It was therefore a surprise to find that CD4$^+$CD8$^-$ thymocytes, a naive population, were capable of protecting a significant proportion of TxX recipients from development of diabetes. Furthermore the relative potency of CD4$^+$CD8$^-$ thymocytes in preventing development of diabetes was found to be far greater than that of peripheral CD4$^+$CD45RC$^-$ cells [14]. Whereas $5 \times 10^6$ CD4$^+$CD45RC$^-$ cells protected all TxX recipients from development of diabetes, half that cell dose protected only 25% of recipients, with no protection observed in recipients of $1.25 \times 10^6$ cells. In contrast, whereas CD4$^+$CD8$^-$ thymocytes protected 11 of 15 TxX recipients at doses of $5 \times 10^6$ cells, significant protection was still observed at cell doses almost one-tenth of this, with two of four TxX rats receiving $6 \times 10^5$ CD4$^+$CD8$^-$ thymocytes failing to develop diabetes. Significantly, this functional potency of CD4$^+$CD8$^-$ thymocytes appears to be restricted only to their ability to prevent diabetes upon adoptive transfer. When assayed for their ability to provide primed B-cells with help for antigen-specific secondary antibody responses, CD4$^+$CD8$^-$ thymocytes were relatively poor at providing such help, in contrast with either naive or primed peripheral CD4$^+$ T-cells ([14]; B. Seddon and D. Mason, unpublished work).

Together, these data suggest that, rather than being generated by clonal expansion peripherally, the cells responsible for protection of

TxX rats from development of diabetes are already generated at a high frequency in the thymus, and it is for this reason that $CD4^+CD8^-$ thymocytes are such a potent source of regulatory cells, a point discussed in some detail elsewhere [14]. In addition, these data yielded two further important results. First, although significant protection from development of diabetes was observed over a wide range of cell doses, at no dose of $CD4^+CD8^-$ thymocytes assayed were all TxX recipients protected from diabetes. Secondly, the dose–response curve for the protection of TxX rats from development of diabetes by $CD4^+CD8^-$ thymocytes was very shallow, only falling from 71% in rats receiving $5 \times 10^6$ cells to 50% of recipients of a tenth of that cell dose. With regard to the first point, the incomplete protection afforded recipients of $CD4^+CD8^-$ thymocytes resembles that of TxX recipients of unfractionated peripheral $CD4^+$ T-cells, in which 50% of rats receiving $10^7$ $CD4^+$ cells were protected [9]. In this case, the incomplete protection of recipients was attributed to an antagonistic effect of $CD45RC^+$ cells on the protective $CD45RC^-$ constituent of the inoculum. It may be therefore that $CD4^+CD8^-$ thymocytes are functionally heterogeneous in a similar manner, containing both regulatory and diabetogenic subsets of cells. This possibility cannot be unreservedly adopted at this time, however, since it is at least theoretically possible that $CD4^+CD8^-$ thymocytes are a functionally uncommitted population that is programmed post-thymically for a variety of roles.

## Theoretical modelling of thymocyte control of autoimmune diabetes

The observed dose–response curve and the possible existence of $CD4^+CD8^-$ thymocytes with the potential to be either regulatory or diabetogenic was further examined by use of a theoretical model. In normal PVG.RT1$^u$ rats, the frequency of regulatory T-cells (designated $T_{reg}$ from here on) that leave the thymus must be sufficiently high to control diabetogenic T-cells ($T_{diab}$) which escape into the periphery, since these rats do not spontaneously develop diabetes. However, in samples of $CD4^+CD8^-$ thymocytes given to recipient TxX rats, the frequencies of $T_{reg}$ and $T_{diab}$ will be subjected to normal sampling variation, and it is therefore conceivable that some inocula will not contain a sufficiently high frequency of $T_{reg}$ to control the $T_{diab}$ constituent. As a result, recipients of such inocula will fail to be protected from development of diabetes and it is this premise that underlies the theoretical analysis [14]. Whereas the data show that $CD4^+CD8^-$ thymocytes are a potent source of regulatory cells, the observation that the peripheral cells that prevent diabetes have the phenotype of a resting memory cell implicates a role for a post-thymic event in their generation. Consequently, as discussed in the previous section, it is an alternative possibility that the $T_{reg}$ and $T_{diab}$ phenotypes develop post-thymically from uncommitted precursors. Although both these hypotheses have quite different physiological implications, the mathematical model is equally applicable to both.

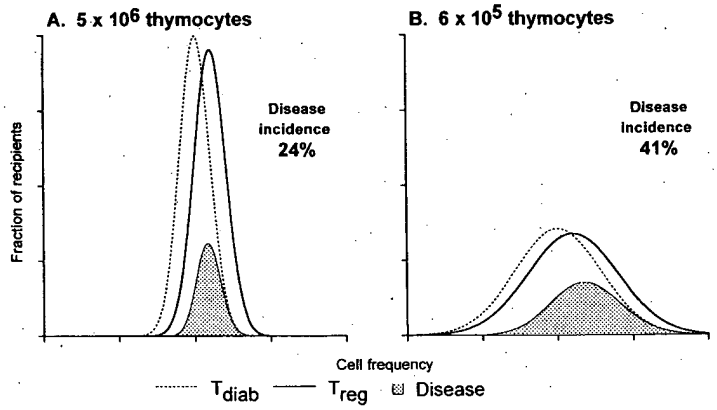
For the purposes of the model, the following simplifications and assumptions were made: (i) the frequencies of $T_{reg}$ and $T_{diab}$ in inocula of $CD4^+CD8^-$ thymocytes are independent normally distributed variables; (ii) inocula containing numerically more $T_{reg}$ than $T_{diab}$ will protect recipient TxX rats from development of diabetes; (iii) the contribution of host $T_{diab}$ is ignored.

On these assumptions, it is possible to determine the distributions of $T_{reg}$ and $T_{diab}$ cell frequencies in different inocula for a given size of $CD4^+CD8^-$ thymocyte cell dose and therefore the probability that recipients thereof will be protected from development of diabetes. Figure 1 shows how the frequencies of $T_{reg}$ and $T_{diab}$ will vary in different inocula for a specific cell dose given to TxX rats. Distributions of these frequencies are shown for cell doses of $5 \times 10^6$ (Figure 1A) or $6 \times 10^5$ (Figure 1B) $CD4^+CD8^-$ thymocytes. Since the $y$-axis represents the proportion of inocula containing a specific frequency of either $T_{reg}$ or $T_{diab}$, the total area under either of these distributions represents all inocula or, by extension, all the recipients of those inocula. Although the expected (i.e. mean) frequencies of $T_{reg}$ and $T_{diab}$ in inocula are unaffected by the size of the cell dose, the breadth (i.e. standard deviation) of their distributions increases with decreasing cell dose size. The shaded curves in Figures 1A and 1B show the predicted proportion of recipients that develop diabetes when given an inoculum of thymocytes containing a specific frequency of $T_{diab}$ cells, calculated for all frequencies of $T_{diab}$. Therefore the areas under these distributions represent the probability that recipients of a given cell dose will not be protected from development of diabetes (Figure 1). Formally, this probability is given by the double integral:

**Figure 1**

**Distributions of the frequencies of $T_{reg}$ and $T_{diab}$ in two different cell doses of $CD4^+CD8^-$ thymocytes**

Graphs show the distributions of $T_{diab}$ (– – –) and $T_{reg}$ (———) in cell doses of $5 \times 10^6$ (A) and $6 \times 10^5$ (B) $CD4^+CD8^-$ thymocytes. The shaded distributions represent the probabilities (*y*-axis) that recipients of inocula of $CD4^+CD8^-$ thymocytes containing different frequencies of $T_{diab}$ (*x*-axis) will develop diabetes. Therefore the area under this distribution represents the fraction of recipients of the specific cell dose of $CD4^+CD8^-$ thymocytes that will not be protected from diabetes development.




$$P = \frac{1}{2\pi}\frac{1}{\mu}\frac{1}{\sqrt{(1+\alpha)}} \times \int_0^{\infty} e^{\frac{-(m-\mu)^2}{2\mu}} \int_0^{m} e^{\frac{-(m-\mu(1+\alpha))^2}{2\mu(1+\alpha)}} \, dm \, dm$$

where $P$ is proportion of rats that receive more diabetogenic cells than regulatory ones, $\mu$ is mean number of diabetogenic $CD4^+CD8^-$ thymocytes in a given sample of these cells, i.e. $\mu = nq$, where $n$ is total number of $CD4^+CD8^-$ thymocytes in the sample and $q$ is the frequency of diabetogenic cells among $CD4^+CD8^-$ thymocytes and $(1+\alpha)$ is the ratio of regulatory to diabetogenic cells among $CD4^+CD8^-$ thymocytes.

In order to calculate $P$, values must be ascribed to the frequencies of $T_{reg}$ and $T_{diab}$. For instance, for a dose of $5 \times 10^6$ thymocytes, in which 27% of recipients were observed to develop diabetes, if $T_{diab}$ are at a frequency of 1:50000, then the model predicts that a mere 10% excess of $T_{reg}$ would result in a disease incidence of 24% of recipients of that cell dose. At higher frequencies of $T_{diab}$, the required excess of $T_{reg}$ is even lower. Significantly, the incidence of disease calculated using these values for the other doses of $CD4^+CD8^-$ thymocytes are in remarkable agreement with the experimental data (Table 1). However, although this obviously does not prove that the underlying assumptions of the analysis are correct, the model does predict that the incidence of diabetes will decrease in recipients of increasing cell doses; specifically, only 6% of recipients of $2.5 \times 10^7$ $CD4^+CD8^-$ thymocytes should develop diabetes. This prediction is currently being tested experimentally (Table 1), and if confirmed, will considerably strengthen the validity of this model.

**Table 1**

**Results of the theoretical analysis of prevention of diabetes by $CD4^+CD8^-$ thymocytes**

The predicted disease incidence was calculated for different doses of $CD4^+CD8^-$ thymocytes using values for the frequencies of $T_{diab}$ and $T_{reg}$ of 1:50000 and 1:45454 respectively. Results are expressed in the 'calculated' column as either a percentage disease incidence or as the expected incidence of diabetes in groups of rats the same size as used experimentally ('observed' column) for a given cell dose of $CD4^+CD8^-$ thymocytes. N.D., not determined.

| Thymocyte dose ($\times 10^6$) | Disease incidence | | | |
|---|---|---|---|---|
| | Observed | | Calculated | |
| 25.0 | N.D. | N.D. | 1/20 | 6% |
| 5.0 | 4/15 | 27% | 4/15 | 24% |
| 2.5 | 5/15 | 34% | 5/15 | 31% |
| 1.25 | 5/12 | 42% | 4/12 | 36% |
| 0.625 | 2/4 | 50% | 2/4 | 41% |

## Phenotypic heterogeneity of $CD4^+CD8^-$ thymocytes

Experimental data and the theoretical analysis of $CD4^+CD8^-$ thymocyte protection of prediabetic TxX rats [14] implies that $CD4^+CD8^-$ thymocytes may contain functionally distinct subsets and raises the possibility that such subsets may also be phenotypically distinct. However, attempts to identify a marker that splits $CD4^+CD8^-$ thymocytes into functionally specialized subsets have been only partially successful. L-Selectin is expressed by approximately half of freshly isolated $CD4^+CD8^-$ thymocytes, and reconstitution of prediabetic rats with either L-Selectin-positive or L-Selectin-negative $CD4^+CD8^-$ thymocytes reveals that the former subset is far more potent than the latter at preventing onset of diabetes [15]. However, only a fraction of recipients were protected, and further studies revealed that the L-Selectin-negative subset, unlike their L-Selectin-positive counterpart, were also incapable of mediating either humoral or cell-mediated response upon adoptive transfer [15]. Given that $CD4^+CD8^+$ thymocytes are L-Selectin-negative and recent thymic emigrants express L-Selectin ([15]; B. Seddon and D. Mason, unpublished work), these data suggest that the L-Selectin-negative subset of $CD4^+CD8^-$ thymocytes is the functionally immature precursor of the L-Selectin-positive population, and that a functional heterogeneity still exists amongst the latter subset. Previous studies have shown that L-Selectin-positive $CD4^+CD8^-$ thymocytes are phenotypically heterogeneous upon activation with respect to expression of class-II antigens and CD8$\alpha$ chain [16], the functional significance of which is under investigation.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